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The role of STING on peroxisome-dependent signalling

O papel da STING na sinalização dependente de peroxissomas

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Thesis submitted to University of Aveiro to fulfil the requirements to obtain the Master degree in Molecular Biomedicine held under the scientific guidance of Dr. Daniela Maria Oliveira Gandra Ribeiro, Invited assistant professor at the Department of Medical Sciences of the University of Aveiro.

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keywords STING, MAVS, RIG-I, cGAMP, cGAS, peroxisomes, innate immunity, IFN, ISG

abstract

Viruses are recognized by several cellular sensors from the innate immune system, activating signalling cascades which result in the production of interferons and other cytokines that affect the virus life cycle and hinder spreading to other cells.

Although the RIG-I/MAVS and the STING pathways are assumed to signal, respectively, for RNA and DNA viruses, there is still some controversy on how these pathways interact with and influence each other. The interaction between STING and MAVS, previously reported to take place at mitochondria, supports a crosslink between these pathways. Our group has recently demonstrated that STING is also able to interact with the peroxisomal MAVS.

With this work we aimed at studying in more detail the interplay between the STING pathway and the peroxisomal RIG-I/MAVS pathway. One of our approaches involved the knock-down of STING and stimulation of the RIG-I/MAVS pathway in cells that contained MAVS solely at peroxisomes, in order to study the importance of STING for the establishment of an effective peroxisome-dependent antiviral response. In parallel, we activated STING by transfecting 2'3'-cGAMP with the objective of performing RT-qPCR analysis of the peroxisome-dependent production of cytokines. The studies initiated with this thesis will contribute to the unravelling of the interplay between the STING pathways and the peroxisomal-dependent RIG-I/MAVS signalling.

Palavras-chave STING, MAVS, RIG-I, cGAMP, cGAS, peroxissomas, imunidade inata, IFN, ISG, PAMP,

resumo

Os vírus são reconhecidos por vários sensores do sistema imunitário inato, responsáveis pela ativação de cascatas de sinalização que levam à produção de interferões e citoquinas, impedindo o ciclo viral e a propagação da infeção às células vizinhas.

Apesar de a via da RIG-I/MAVS e da STING serem respetivamente responsáveis pelo reconhecimento de vírus de ARN e ADN, existe ainda alguma controvérsia sobre como estas duas vias interagem. A interação entre a STING e a MAVS, anteriormente reportada nas mitocôndrias, sugere uma interligação entre as duas vias. O nosso grupo demonstrou recentemente que existe também uma interação entre a STING e a MAVS peroxisomal.

Neste trabalho, o nosso objetivo consistiu em estudar a interligação entre a via da STING e a via RIG-I/MAVS peroxisomal. Começamos por silenciar a STING e a estimular a via RIG-I/MAVS numa linha celular que contem MAVS apenas nos peroxissomas, para estudar a influência da STING na resposta antiviral dependente dos peroxissomas. Por outro lado, tentamos ativar a STING através da transfeção da molécula 2'3'-cGAMP com o objetivo de analisar a produção de citoquinas e interferões dependentes da via peroxissomal por RT-qPCR. As experiências apresentadas nesta tese irão certamente contribuir para desvendar a interligação entre a via da STING e a via RIG-I/MAVS dependente dos peroxissomas.

List of abbreviations

aa	amino acids
CARD	caspase activation and recruitment domain
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cyclic GMP-AMP synthase
DNA	deoxyribonucleic acid
ds	double-stranded
ER	endoplasmic reticulum
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
IRF	interferon regulator factor
IFNs	Interferons
IKK	inhibitor of κB kinase
ISG	interferon-stimulated gene
KD	knock-down
MAM	mitochondria-associated membranes
MAVS	mitochondrial antiviral signalling
MDA5	melanoma differentiation-associated gene-5
MEF	mouse embryonic fibroblasts
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cell
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I like receptors
RNA	ribonucleic acid
RNAi	interference RNA
RNF	RING finger protein
RT-qPCR	Quantitative Reverse Transcription Real Time Polymerase Chain Reaction
ss	single-stranded
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TBK1	TANK-binding kinase 1
TRAF	tumor necrosis factor (TNF) receptor-associated factor
ULK1	UNC-51-like kinase
vMIA	Viral Mitochondria-localized Inhibitor of Apoptosis

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I. Introduction

I. Introduction

1.1 – Innate Immune Responses against Viral Infections

The innate immune system holds very well defined mechanisms to defend the host against invading pathogens. These are composed by pattern-recognition receptors (PRRs), molecules that recognize pathogen-associated molecular patterns (PAMPs) that are specific from each invader (Janeway, 1989; Lemaitre et al., 1996). The 3 most known types of PRRs are: Toll-like receptors (TLRs), RIG-I–like receptors (RLRs) and NOD-like receptors (NLRs). TLRs and RLRs are activated by nucleic acids from microbes (Ran et al., 2014; Sun et al., 2009). While RLRs identify mainly viruses, NLRs are specialized in bacterial recognition (Franchi et al., 2010) and TLRs recognize both types of pathogens. Interferons (IFN) and other cytokines are produced due to the binding of PAMPs to these PRRs triggering signalling pathways that allow the effective innate defence of the host and also the stimulation of the adaptive immune response (Biacchesi et al., 2012).

1.1.1 – Cytosolic RNA sensors and the MAVS pathway

RLRs such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) are cytosolic receptors that recognize abnormal cytosolic RNA (Cai et al., 2014; Ran et al., 2014; Yoneyama and Fujita, 2008). RLRs are composed by a DExD/H-box RNA helicase domain and two N-terminal caspase recruitment domains (CARD) (Biacchesi et al., 2012; Sun et al., 2009). The helicase domain is crucial for sensing and binding to pathogen nucleotides and the N-terminal CARDs play an important role in the production of type I IFN, inducing the downstream signalling cascade (Sun et al., 2009). Experiments with overexpression of solely the RIG-I CARD domain demonstrated that this region by itself could lead to the activation of the antiviral response and production of IFN (Ferreira et al., 2016; Magalhães et al., 2016; Yoneyama et al., 2005). RIG-I and MDA-5 are distinct as they sense different types of RNAs: RIG-I identifies single-stranded RNA and short double-stranded RNA that have a 5’ triphosphate group, while MDA-5 recognizes long double-stranded RNAs (Kato et al., 2008; Takeuchi and Akira, 2010). Viral double-stranded RNA viruses can also be recognized by TLRs, membrane-bound receptors that are present at the plasma membrane and endosomes (Yoneyama and Fujita, 2008). RLRs are capable of identifying an extensive diversity of positive- and negative-stranded RNA viruses and, indirectly, DNA viruses if RNA polymerase III is used (Biacchesi et al., 2012; Ferreira et al., 2016; Franchi et al., 2010; Ran et al., 2014).

Inactive RIG-I is stabilized in the C-terminal by a repressor domain (RD) in order to suppress CARDs. When the cell is infected, this RD is released upon detection of exogenous

RNA and RIG-I is activated being able to interact with the mitochondrial antiviral signalling (MAVS) protein (also known as IPS-1, Cardif or VISA), its downstream adaptor molecule (**Figure 1**) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

MAVS holds a structure that allows it to bind with upstream signalling molecules such as RIG-I through its N-terminal CARD-like domain, its C-terminal transmembrane domain facilitates the attachment to the organelle membranes and it also contains a proline-rich sequence useful for protein-protein interactions (Belgnaoui et al., 2011; Seth et al., 2005). MAVS is expressed in the outer mitochondrial membrane, peroxisomes and mitochondria-associated membranes of the endoplasmic reticulum (MAM) (Dixit et al., 2010; Horner et al., 2011; Seth et al., 2005). RIG-I activates MAVS in the presence of viral RNA and induces its transformation into prion fibre-like active aggregates (Hou et al., 2011). It was shown that the N-terminal CARD domain was enough to produce these functional aggregates (Xu et al., 2014). After being activated, this virus-induced signalling adapter triggers two kinase complexes, the TANK-binding kinase-1 (TBK1)/IκB kinase I and the initiation κB kinase (IKK), to induce the phosphorylation of transcription factors IRF3/7 leading to the production of type I IFNs and induction of NF-κB which stimulates activation of proinflammatory cytokines (Akira et al., 2006; Castanier et al., 2010; Sun et al., 2009; Yoneyama and Fujita, 2009).

MAVS goes through several post translational modifications such as ubiquitination and phosphorylation which are important to maintain the balance in the signalling pathway since they allow the cell to shut down the IFN antiviral response (Belgnaoui et al., 2011; Vazquez and Horner, 2015; You et al., 2009).

There is currently some controversy concerning the distinct roles of MAVS on the different organelles. Kagan's group supports that activation of mitochondrial MAVS mainly leads to the production of type I IFN and IFN-stimulated genes (ISGs), whereas peroxisomal MAVS triggers the induction of ISGs and type III IFN (Dixit et al., 2010; Odendall et al., 2014). However, Bartenschlager's group defends that both MAVS lead to the production of type I and III IFN with similar efficiency (Bender et al., 2015). Mitochondrial MAVS seems to generate a less quick but long-lasting response being dependent on IFNs while peroxisomal MAVS leads to a faster and more transient reaction IFN-independent response (Dixit et al., 2010; Islinger et al., 2012).

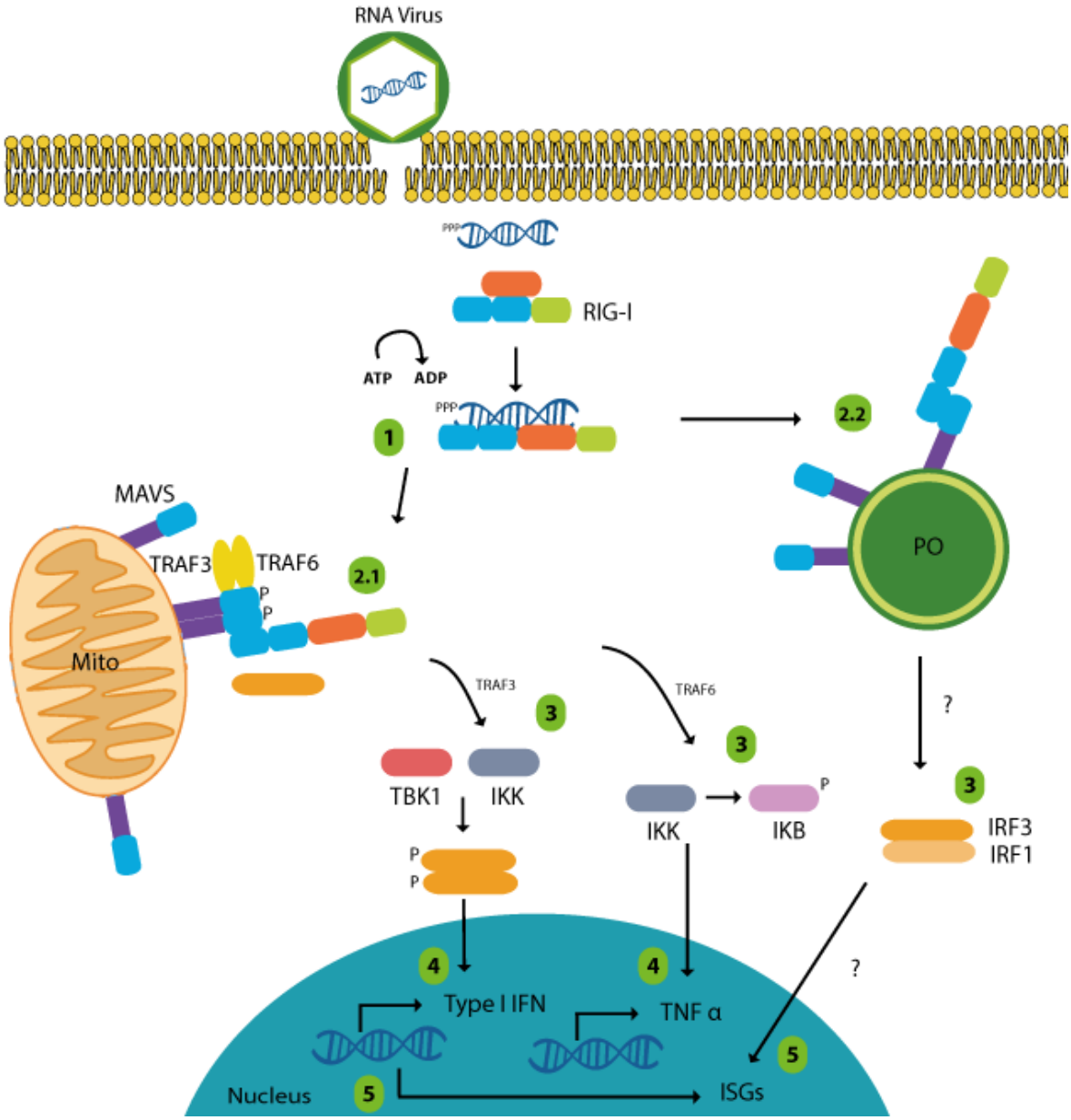

Figure 1 – RNA recognition by RIG-I. Viral RNA is sensed by RIG-I which is ubiquitinated and hydrolyses ATP to change its conformation [1] in order to interact with MAVS at mitochondria [2.1] and peroxisomes [2.2]. This interaction occurs via the CARD domains of both protein. Following RIG-I binding, mitochondrial MAVS polymerizes and recruits TRAF proteins, TRAF3 and TRAF6, to activate IRF3 kinases TBK1/IKKε which in turn phosphorylate MAVS [2.1]. Phosphorylated MAVS recruits IRF3 for phosphorylation by TBK1 and IKK phosphorylates IKB to activate NF-kB pathway [3] (Liu et al., 2015a). Phosphorylated IRF3 and activated NF-κB are translocated to the nucleus where they induce type I IFN [4] which triggers the transcription of genes implicated in the antiviral response (ISGs) [5] and other proinflammatory cytokines [4] (Arnoult et al., 2009). Peroxisomal RIG-I-MAVS interaction

leads to IRF1 and 3 activation [3] which translocate to the nucleus and generate ISGs [5] (Ferreira et al., 2016; Kell and Gale, 2015; Liu et al., 2016). Mito: mitochondria; PO: Peroxisome.

1.1.2 – Cytosolic DNA sensors and the STING pathway

Besides exogenous RNA, eukaryotic cells are also able to detect non-self DNA in the cytosol and produce an effective antiviral signaling response (Barbalat et al., 2011). The endoplasmic reticulum (ER)-resident transmembrane protein stimulator of IFN genes STING (also known as MITA, mediator of IRF3 activation; ERIS, endoplasmic reticulum interferon stimulator and MPYS, N-terminal methionine-proline-tyrosine-serine protein) plays a main role in this signalling pathway by inducing the expression of type I IFNs and other cytokines (Barber, 2011; Biacchesi et al., 2012). STING is present in several tissues and exists in different cells such as macrophages, dendritic cells, T cells, endothelial and epithelial cells and fibroblasts (Campos et al., 2014; Ishikawa and Barber, 2008).

STING is a 42kDa protein and has been shown to be mainly inserted in the endoplasmic reticulum (ER) and to interact with RIG-I, MAVS, TBK1 and IKKε (Ishikawa and Barber, 2008; Nitta et al., 2013). STING contains 4 transmembrane domains in the N-terminal (1- 137 aa) (Ran et al., 2014; Sun et al., 2012). The globular C-terminal domain (138-379 aa) projects to the cytosol to interact with cyclic-dinucleotides (CDNs) and to trigger the downstream signalling. This domain has a C-terminal tail that is responsible for the auto-inhibition of STING (Campos et al., 2014). The N-terminal domain is essential for its correct activation and translocation (Poltorak et al., 2016).

1.1.2.1 – Activation of the STING pathway

STING has been shown to play an important role on the innate immune responses to DNA viruses, several RNA viruses, retroviruses, bacteria and protozoan parasites. Furthermore, it is also involved in inflammatory and autoimmune diseases that are developed because this system detects abnormalities in its self-DNA that becomes loose in the cytosol (**Figure 2**) (Ahn et al., 2012; Gao et al., 2013; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Kondo et al., 2013; Sauer et al., 2011; Sharma et al., 2011; Sun et al., 2009; Watson et al., 2012).

STING is able to activate the expression of type I IFN and proinflammatory cytokines through four distinct mechanisms identified until now. It may function as an adaptor of DNA and RNA sensors, functioning downstream of several DNA sensors (e.g. DDX41 and IFI16) or sensing RNA viruses; it may directly interact with CDNs from bacteria or produced by the DNA sensor cyclic GMP-AMP Synthase (cGAS); it may be activated by RIG-I pathway

when this is triggered by an RNA agonist involving an autocrine/paracrine mechanism; or it may act as a sensor of membrane fusion (Holm et al., 2016; Liu et al., 2016; Ouyang et al., 2012; Sun et al., 2009).

Several studies show that STING interacts directly with cytosolic DNA and that it is involved in the innate immune response to RNA but not as a receptor itself (Nazmi et al., 2012). Experiments with poly(I:C) proved that STING does not bind to it. Therefore, up to now it has been assumed that STING acts through RIG-I pathway, interacting with MAVS at the mitochondria, and assembles the signalling complex STING-TBK1-IRF-3 (Abe et al., 2013; Maringer and Fernandez-sesma, 2014).

There are several DNA sensors, such as DAI, IFI16, DHX36, DHX9, DDX41, but recently the DNA sensor cGAS (also known as MB21D or C6orf150) emerged and genetic studies suggest that it has a key role in the antiviral response (Ran et al., 2014; Rathinam and Fitzgerald, 2011; Schoggins et al., 2014). Ishikawa's group showed that STING-deficient cells could not express IFNs when invaded by dsDNA (Ishikawa et al., 2009). It was also demonstrated the presence of a second messenger, cGAMP, when cells were infected with DNA viruses or transfected with DNA (Gao et al., 2013). This molecule is produced by cGAS in a DNA-dependent manner. When cGAS recognizes possible pathogen DNA or self-DNA it synthesizes cGAMP that interacts with STING and activates it in order to start the IFN antiviral response (Sun et al., 2013). After binding to STING, cGAMP assumes a closed conformation. The mechanisms through which cGAMP binds to STING and how it triggers STING dimerization are still unknown (Shu et al., 2014).

Besides recognizing exogenous DNA, cGAS may also sense RNA. Some recent studies propose a distinct way through which RNA viruses are recognized that does not depend on RIG-I signalling (Maringer and Fernandez-sesma, 2014). Schoggins' group demonstrated that cGAS is a strong protector against all the positive-sense RNA they analysed even when RIG-I was not present (Schoggins et al., 2014). Therefore, it is not known yet the mechanism through which cGAS recognizes RNA and the reason for its specificity for positive-sense RNA viruses (Maringer and Fernandez-sesma, 2014). Schoggins et al. defend that cGAS is a fundamental molecule in the cellular immune response against both DNA and RNA viruses (Schoggins et al., 2014).

However, a new and unexpected model arose supported by Liu et al. in which DNA virus infection is ceased by activation of the RIG-I pathway with an RNA agonist. This allows the induction of STING expression in order to fight the virus more efficiently. Against what could be thought until now, this group supports that STING is not only activated by cGAMP but also by several downstream factors of the RIG-I pathway. It was seen that STING production is mediated by STAT and NF-κB pathways involving the synergistic activities of type I IFN and TNFα, respectively. Therefore, this is dependent on an autocrine/paracrine mechanism. A deficit in any of these pathways leads to an inefficient DNA viral protection since this crosstalk signalling is not able to activate STING expression (Liu et al., 2016).

Recently, a new mechanism was proposed for STING signalling activation which is related with membrane fusion events upon viral invasion. Holm et al. reported that a STING-dependent but cGAS-independent pathway exists and that it is activated by lipid membrane fusion. Therefore, the process of merging of the host cell membrane with the viral membrane promotes type I IFN production. The authors identified this novel model of STING activation in response to enveloped RNA viruses, specifically Influenza A virus (Holm et al., 2016).

Activation of STING involves its dimerization: in physiological conditions, STING is usually monomeric but the presence of dsDNA or dsRNA was proven to drive its dimerization (Sun et al., 2009). Upon activation, STING travels to the perinuclear area where it acts as a scaffold protein, making punctate vesicles in which the interaction between STING, TBK1 and IRF3 is favoured. The following phosphorylation of STING and IRF3 by TBK1 results in the traffic of IRF3 to the nucleus and finally the stimulation of type I IFN and other cytokines (Ran et al., 2014). Besides TBK1, STING also interacts with the kinase IKK which induces the transcription factor NF-kB that translocates to the nucleus and works with IRF3 to stimulate IFNs and proinflammatory cytokines (Wu et al., 2013). STING dimerization and the assembly between STING and TBK1 are thought to be induced by tripartite motif-containing 32 (TRIM32) and TRIM56 that bind to STING and mark it for K63-linked ubiquitination. Therefore, TRIM32 and TRIM56 are interferon-inducible E3 ubiquitin ligases that have the ability to modify STING in order to produce a signal towards cytosolic nucleic acids (Maringer and Fernandez-sesma, 2014; Tsuchida et al., 2010). TRIM38 is also an ubiquitin ligase involved in the activation of STING pathway. cGAS and STING are both sumoylated by TRIM38 in uninfected cells and during the early phase of viral infection. Hu et al. demonstrated that cGas sumoylation avoided its polyubiquitination and degradation and that STING sumoylation allowed its activation and stability during early stage viral infection (Hu et al., 2016). However, in the late phase of infection these two proteins were shown to be desumoylated by Senp2 which led to their degradation. This system guarantees an efficient immune antiviral response to DNA virus in the early phase of infection and stops the activation signal in the late stage in order to balance the immune reaction (Hu et al., 2016).

The activation of STING is not completely understood in the structural level since reports could not yet disclose several parameters such as the data on full-length STING, the reason for its localization and to view and analyse the C-terminal tail in crystal structures. However, it is known that it travels to the perinuclear area generating vesicles that support signalling molecules interactions and originate the innate immune response (Cai et al., 2014).

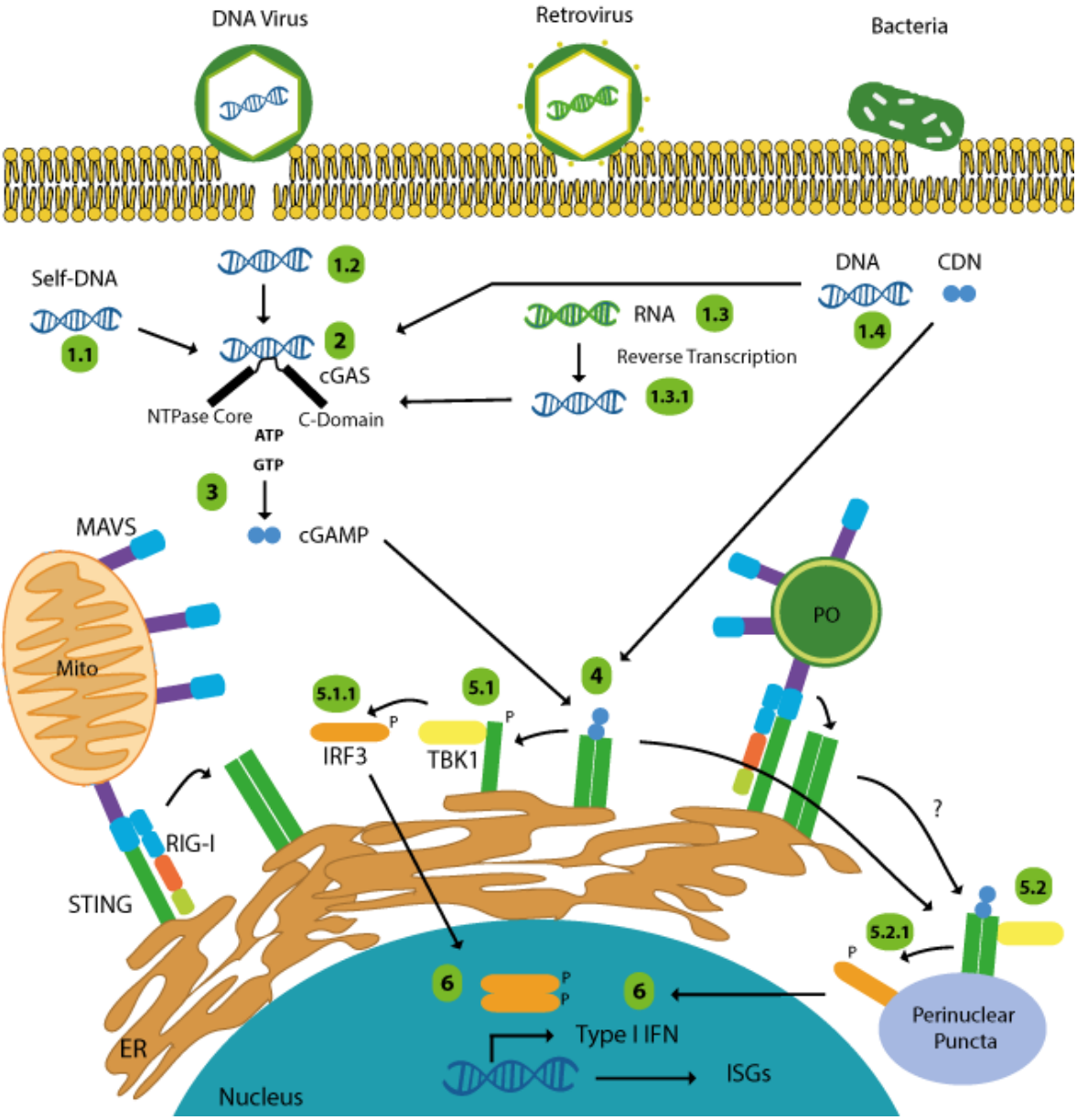

Figure 2 – STING role in the innate immune response towards cytosolic nucleic acids. STING pathway may be activated through several mechanisms: self-DNA released from mitochondria or in the cell destruction process [1.1]; viral DNA [1.2] or RNA [1.3] infection; bacterial infection [1.4]. Cytosolic DNA is recognized by cGAS [2] and the interaction between them result in the production of 2'-3'-cGAMP [3]. 2'-3'-cGAMP binds to STING in the ER which provokes STING dimerization [4]. STING may travel to the perinuclear region forming punctate vesicles [5.2] that contain IRF3 and TBK1 which favours TBK1-IRF3 interactions and phosphorylation of IRF3 by TBK1 [5.2.1]. This process activates IRF3 which traffics to the nucleus as a dimer where it induces type I IFNs and inflammatory cytokines [6] (Ishikawa et al., 2009; Ran et al., 2014). STING may also remain in the ER [5.1] where it might be phosphorylated by TBK1 to recruit IRF3 and TBK1 in turn phosphorylates IRF3 [5.2.1], which travels to the nucleus as a dimer and triggers type I IFNs and inflammatory cytokines [6] (Liu et al., 2015a). Besides, cytosolic DNA, c-di-GMP and c-di-AMP from

bacteria may also induce the innate immune response binding directly to STING [4]. Additionally, retroviruses may also lead to the generation of cGAMP through the production of cDNA during reverse transcription which is recognized by cGAS [1.3.1] (Ran et al., 2014). In case of RNA being present, RIG-I is activated (not shown) and binds to MAVS at mitochondria and this complex might interact with STING stimulating the STING downstream signalling [5.2] (Zhong et al., 2008). Mito: Mitochondria; ER: Endoplasmic Reticulum.

1.1.2.2. – Regulation of the STING pathway

Besides the ubiquitination responsible for STING positive regulation that allows it to dimerize and thereafter to be activated, as described above, STING may also be modulated negatively by ubiquitination leading to its degradation. This molecule is also up and downregulated by phosphorylation (Diner and Vance, 2014; Ran et al., 2014). Therefore, STING may undergo K48-linked ubiquitination by RNF5, an E3 ubiquitin ligase, in the lysine 150 which drives it for degradation (Zhong et al., 2009). Another E3 ligase responsible for this process is RNF26 that does the k11-linked polyubiquitination in the same residue. This enzyme weakens the ubiquitination made by RNF5 indicating a possible role in the positive regulation of STING (Qin et al., 2014).

Beyond ubiquitination, STING may also go through phosphorylation in order to activate the signaling pathway. It is known that STING is phosphorylated by TBK1 following cGAMP binding, but no other enzymes had been described with this function. This post-translational modification of SER and THR residues of STING is led by the changes in the dynamics of its CTT tail caused by cGAMP interaction (Tsuchiya et al., 2016). This pathway has also several mechanisms of downregulation by phosphorylation. Degradation of STING can occur when it is negatively modulated by S366 phosphorylation. Though, Konno and his group identified 272 enzymes and reported that the S366 in the C-terminal domain of STING may be phosphorylated by the autophagy-associated kinase UNC-51-like kinase (ULK1). They suggest a model in which STING is negatively modulated by ULK1 phosphorylation in the S366 residue, adding that the negative influence on STING pathway does not depend on autophagy but rather on direct phosphorylation (Konno et al., 2013). Usually mammalian Target of Rapamycin (mTOR) or AMP-activated protein kinase (AMPK) regulate pathways responsible for the phosphorylation of ULK1 which keep it in an inactive condition. Konno and his team report that ULK1 is activated by cGAMP and it does not depend on STING, although the mechanism through which cGAMP influences ULK1 is yet unknown (Diner and Vance, 2014). Besides this process of regulation there are others already studied, such as autophagy and the detection of intracellular DNA by the 3' repair exonuclease 1 (TREX1) that negatively modulate STING (Gall et al., 2012). The autophagy modulator, Beclin-1, was shown to interact with cGAS which prevents STING activation

when infection occurs. This mechanism allows the cell to balance the immune response (Ma and Damania, 2016). Phosphodiesterases are also thought to be important for STING regulation as they regulate the amount of cyclic dinucleotides present in the cell, although evidence is necessary to understand this modulation and other possible pathways of regulation (Diner and Vance, 2014).

It is not clear yet whether there is contribution of other post-translational alterations that regulate this pathway and how STING is efficiently activated to protect the organism of DNA pathogens. Besides, it is not understood how this process is shut down at the right time so that the immune response is not self-destructive.

STING was also reported to interact with and be regulated by viral proteins: several viral proteins bind to STING in order to block its signalling and consequently the IFN response (Aguirre et al., 2012; Ding et al., 2013; Holm et al., 2016; Lau et al., 2015; Liu et al., 2015b; Ma et al., 2015a; Sun et al., 2012).

1.1.3 – Interactions between the STING and MAVS pathways

In 2008, Ishikawa and Barber and Zhong et al. identified the interaction between STING and both MAVS and RIG-I. Both groups proved that STING acts downstream of the MAVS pathway, showing that overexpression of STING leads to activation of IRF3 even when MAVS is not present (Ishikawa and Barber, 2008; Zhong et al., 2008). These interactions, as described above, are essential for propagation of signalling cascades induced by foreign nucleic acids. However, these two manuscripts strikingly differ in what the localization of STING is concerned. Ishikawa and Barber report that STING is found in the endoplasmic reticulum while Zhong et al. find it in the mitochondrial membrane. A paper from the same year by Jin et al. identifies STING in mitochondria and in the plasma membrane (Jin et al., 2008).

Ishikawa and Barber assume that STING may be an important downstream adaptor molecule that facilitates RIG-I and MAVS function but they did not clarify whether MAVS directly interacts with STING or exists as a complex with RIG-I/STING. Hereupon, Zhong et al. conclusions are more straightforward. The authors propose a model that defends the formation of a complex between RIG-I, MAVS and STING triggered by the binding of nucleic acid to RIG-I upon viral infection. This complex then recruits TBK1 to phosphorylate IRF3 which then directly activates IFN transcription. The regulatory changes (e.g., post-translational modifications) that occur in all these molecules upon binding to this complex still need to be determined (Zhong et al., 2008).

Maringer et al. present an overview of the ways in which STING facilitates sensing of RNA viruses. These include modulation of RIG-I-dependent responses through STING's interaction with MAVS, and more speculative mechanisms involving the DNA sensor cGAS

and sensing of membrane remodelling events. An enlightening figure of these pathways is presented by Maringer et al., where it is shown that viral RNA is recognized by RIG-I, which associates with MAVS and STING at MAMs to activate STING, and after its translocation to perinuclear vesicles, STING then acts as a scaffold for the recruitment of TBK1 and other signalling components required for IRF3 activation and type I IFN induction (Maringer and Fernandez-sesma, 2014).

1.2 – Peroxisomes' role in the cellular innate immune response

1.2.1 - Peroxisomes

1.2.1.1 – Structure, Location and Biogenesis

In 1954, Johannes Rhodin discovered peroxisomes in mouse kidney through electron microscopy. They were termed microbodies until De Duve and Baudhuin in 1966 discover that they co-localized with hydrogen peroxide (H_2O_2-producing oxidases, catalases and an H_2O_2 -degrading enzyme), being named peroxisomes after it (Schrader and Fahimi, 2008).

Peroxisomes are spherical single-membrane-bound organelles that are spread throughout the cytoplasm of most eukaryotic cells (Camoes et al., 2009; Lodhi and Semenkovich, 2014; Mast et al., 2015; Schrader et al., 2012). Their shape and size differ depending on the tissue they are localized, varying from 0.1 to 0.5 µM in diameter and on the environmental modifications that might force them to adapt their function, number and morphology (Lodhi and Semenkovich, 2014; Schrader and Fahimi, 2008). Peroxisomes have, hence, high plasticity and dynamics (Smith and Aitchison, 2013).

Peroxisomes are free of DNA and protein synthesis, having most of its proteins produced on free polyribosomes in the cytosol (Camoes et al., 2009). Peroxisomes' proteins are inserted into the organelle through an exclusive method different from the introduction system into the ER, mitochondria or chloroplasts, as they can hold proteins that are completely folded, that are oligomeric and that contain a cofactor. This is possible because peroxisomes have shuttling receptors which send proteins from the cytoplasm to the peroxisome matrix and then return again to the cytoplasm to be reused (Dammai et al., 2001). These receptors are soluble proteins, peroxins (Pex), which bind to a docking site at the peroxisomal membrane forming a channel through which cargo can be loaded. Most of the peroxins known up to date are implicated in the import of proteins to the matrix (e.g. Pex5p). Regarding the transport of peroxisomal membrane proteins, , another type of machinery is used that mainly involves Pex19p (Schrader et al., 2012).

Peroxisomes have, at least, two distinct and likely complementary biogenesis pathways (**Figure 3**). They can be formed by growth and division of pre-existing

peroxisomes and they can arise by de novo budding from the ER (Smith and Aitchison, 2013). Growth and division of the organelle implicates its elongation, constriction and fission, which, in mammals, involves the adaptor proteins Fis1 (Fission 1) and mitochondrial fission factor (MFF) as well as the large and self-assembling GTPases dynamin-like (-related) proteins (DLPs/DRPs), which generate spiral-like arrangements around constricted membranes with the purpose to facilitate membrane segregation (Praefcke and McMahon, 2004; Schrader et al., 2012). These proteins are also present in mitochondria, being part of the division machinery of both organelles (Islinger et al., 2012). De novo synthesis from ER might involve the budding of biochemically different preperoxisomal vesicles from the ER and their fusion, comprising at least two of these vesicles. Each of these vesicles has half a peroxisomal translocon complex resulting in one functional translocon upon fusion, being able to import enzymes from the cytoplasm (van der Zand et al., 2012).

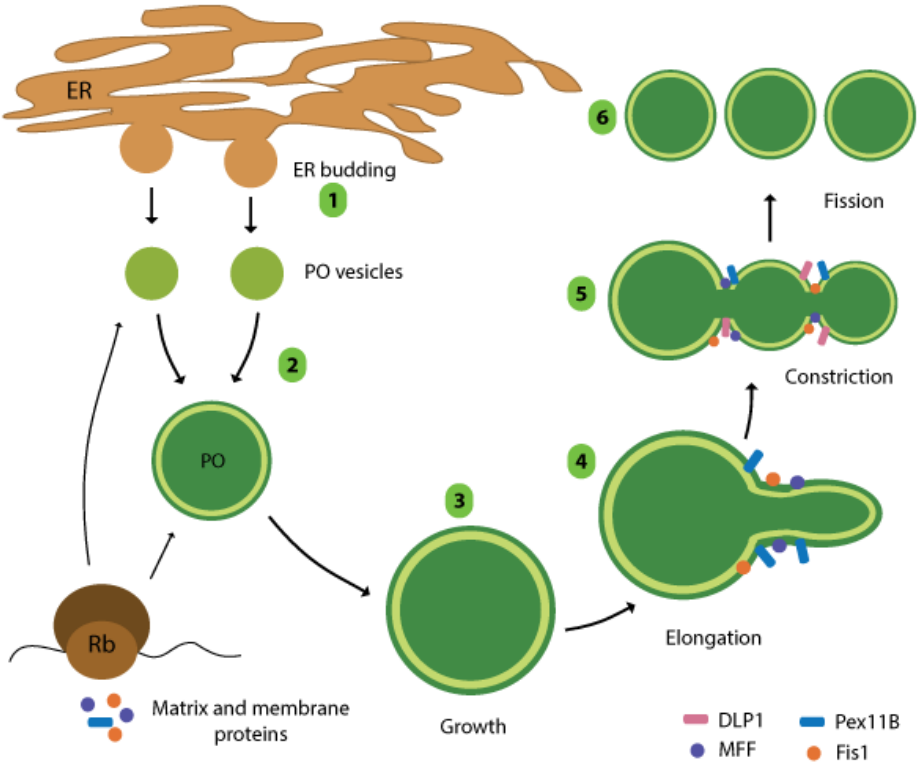

Figure 3 – Peroxisome formation in mammalian cells. Peroxisomes can be originated through budding of vesicles from the ER [1]. At least, two different pre-peroxisomal vesicles fuse and a mature peroxisome, able to import membrane and matrix proteins, is formed [2]. Peroxisomes can also multiply by growth [3] and division. A mature peroxisome suffers a sequence of morphological modifications, such as membrane elongation, constriction and fission. A tubular membrane prolongation is initiated by Pex11β and several specific peroxisomal membrane proteins are recruited, such as Fis1 and MFF [4]. These proteins concentrate at constriction sites and MFF recruits DLP1 to favour membrane fission [5]. DLP1 forms large self-assembling ring-like structures. Pex11β activates DLP1 GTPase

activity which drives to the constriction of its ring and, ultimately, to peroxisome scission [6] (Schrader et al., 2016; Smith and Aitchison, 2013). PO: Peroxisome; ER: Endoplasmic Reticulum; Rb: Ribosomes.

1.2.1.2 - Functions

Peroxisomes have several distinct functions depending on the species, tissue and environmental stimuli. They are involved in metabolic and antiviral pathways and in cellular homeostasis (Schrader et al., 2012). They play an important role on the oxidation of lipids, especially fatty acids, and the metabolism of H_2O_2 since they oxidise substrates, such as lactate, glycolate, oxalate, urate, producing H_2O_2 and decomposing it using catalases. Regarding lipid metabolism, peroxisomes are in charge of the α- and β-oxidation of fatty acids (being the solely responsible for the β –oxidation of very long chain fatty acids), fatty acid elongation and synthesis of ether phospholipids, such as plasmalogens, in mammals which compose the neuronal myelin sheath (Camoes et al., 2009). Another set of specialised functions involve penicillin production in fungi, glycolysis in protozoa, photorespiration and the glyoxylate cycle in plants. Moreover, peroxisomes are also implicated in the production of bile acids and docosahexaenoic acid, an omega-3 fatty acid which is a modulator of neuronal function, in the metabolism of amino acids, catabolism of purines, polyamines and prostaglandins and eicosanoids, which are intermediaries of inflammation (Schrader and Fahimi, 2008; van den Bosch et al., 1992; Wanders and Waterham, 2006).

If peroxisomes functions suffer a failure, toxic substances might accumulate, such as phytanic acid and very long chain fatty acids, and essential peroxisomal products decrease, highly compromising human life sustainability (Camoes et al., 2009).

1.2.2 – Peroxisomes and antiviral signalling

The exciting discovery that peroxisomes, in concert with mitochondria, act as signalling platforms in antiviral defence (Dixit et al., 2010), conferred them a novel function, highlighting their important role in health and disease. Although very promising, the relationship between viruses and peroxisomes has not yet been studied in detail.

Ribeiro's group has shown that both Human cytomegalovirus (HCMV) and Hepatitis C virus (HCV) have developed specific mechanisms to evade the peroxisome-dependent antiviral response (Ferreira et al., 2016; Magalhães et al., 2016). HCMV encodes Viral mitochondria-localized inhibitor of apoptosis (vMIA), a protein that, besides playing an important role on the inhibition of apoptosis, has been shown to interfere with the mitochondria-dependent antiviral signalling pathway (Castanier et al., 2010; McCormick et

al., 2003). Magalhães et al have shown that vMIA is also localized at peroxisomes, interacts with MAVS and specifically inhibits the cellular antiviral response established at this organelle (Magalhães et al., 2016). It furthermore induces peroxisomal fragmentation via a mechanism that seems to be distinct from the one occurring in mitochondria (Magalhães et al., 2016). Ferreira et al have also demonstrated that the NS3-4A protein from HCV is able to cleave the peroxisomal MAVS, dissociating it from the organelle and blocking downstream signalling (Ferreira et al., 2016).

II. Objectives

II. Objectives

The nucleic acid composition of each virus strongly influences the cellular antiviral response. The RIG-I/MAVS and the STING pathways are assumed to signal, respectively, for RNA and DNA viruses. However, it has been suggested that these pathways may interact and influence each other at some point.

STING has been shown to interact with RIG-I and mitochondrial MAVS, playing a role on the mitochondrial-dependent signalling pathway. Our group has recently demonstrated that STING is also able to interact with the peroxisomal MAVS. This interaction occurs even in the presence of HCMV's vMIA, a viral protein that interferes with the interaction between STING and mitochondrial MAVS, suggesting once more the existence of important differences between the antiviral pathways established at these two organelles.

The main objective of this study is evaluating whether and how the STING pathway influences the peroxisomal RIG-I/MAVS pathway. To that end, two distinct approaches were planned:

- Analysis of the importance of STING for the establishment of an effective peroxisomal MAVS-dependent antiviral response

- Analysis of the peroxisomal MAVS-dependent antiviral response upon stimulation of the STING pathway

III. Materials and Methods

III. Materials and Methods

3.1 - Materials

Antibodies

Western Blotting

Primary

- rabbit anti-RIG-I (1:200, Santa Cruz Biotechnology)
- rabbit anti-STING (1:500, Cell Signaling)
- mouse anti-α-Tubulin (1:4000, Sigma-Aldrich)

Secondary

- anti-rabbit IR Dye 800 CW (1:10000, Li-Cor)
- anti-mouse IR Dye 680 RD (1:10000, Li-Cor)

Cells strain

- Mouse Embryonic Fibroblasts (Mefs) MAVS-PEX cells

Chemicals and reagents

- 2'3'-cGAMP, Invivogen
- Acetic Acid, Merck Millipore
- Acrilamide, Fisher Scientific
- Agarose, Roth
- Ammonium Persulfate (APS), Sigma
- Bovine serum albumin (BSA), NZYTech
- Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad
- Bromophenol Blue, Sigma
- Dithiothreitol (DTT), Sigma
- Ethanol, Merck Millipore
- Ethylenediaminetetraacetic acid (EDTA), Sigma
- Foy, Schwarz-Pharma
- Glucose, Fluka
- Glycerol, Roth
- Glycine, Fisher Scientific
- Hydrochloric acid (HCl), Merck Millipore
- Isopropanol, Merck Millipore
- Methanol, Merck Millipore
- Milk, Néstle

- Potassium chloride (KCl), Sigma
- Penicillin/ Streptomycin, BioWest
- Phenylmethylsulfonyl fluoride (PMSF), Sigma
- Sodium chloride (NaCl), Sigma
- Sodium dodecyl sulfate (SDS), Sigma
- Sodium Deoxylacholat, Sigma
- Sodium phosphate (NaHPO4), Sigma
- STING siRNA, Invitrogen
- Tetramethylethylenediamine (TEMED), Fluka
- Tris, Fischer Scientific
- Trasylol, Bayer
- Triton, Sigma
- Tween-20, Sigma
- β-Mercaptoethanol, Sigma

Culture cell solutions and plates

- Dulbecco's Modified Eagle Medium (DMEM) High Glucose w/ L-Glutamine w/o Sodium Pyruvate, BioWest
- Dulbecco's Phosphate Buffered Saline w/o Calcium w/o Magnesium, BioWest
- Trypsin-EDTA 1X in PBS w/o Calcium w/o Magnesium w/o Phenol Red, BioWest
- Fetal Bovine Serum (FBS), qualified, E.U.-approved, South America origin, BioWest
- Opti-MEM Reduced-Serum Medium (1x) liquid, Gibco

Databases and Software

- Quantity One 1-D Analysis Software, Bio-Rad
- Excel, Microsoft
- National Center for Biotechnology Information (NCBI)
- Image Studio Software for Odyssey
- Axio Imager software

Equipment

- Centrifuge Heraeus Pico and Fresco 17, Thermo Scientific
- UV-3100 PC Spectrophotometer, VWR
- Vacuum gas pump, VWR
- Shaker, Mini-Rocker PMR-30, Grant Bio
- Pipettes Eppendorf Research, Eppendorf
- Basic pH meter PB-11, Sartorius
- CO_2 incubator MCO-17AIC, Sanyo
- Thermomixer Comfort 1.5, Eppendorf

- PowerPac HC High-Current Power Supply, Bio-Rad
- Mini protein Tetra Cell and blotting module, Bio-Rad
- Dry block thermostat, Grant
- DS-11 Spectrophotometer/Fluorometer Series, DeNovix

Kits
- NucleoBond® Xtra Midi, Macherey-Nagel

Marker
- NZYColour Protein Marker II, NZYtech

Membranes
- Protran BA85 Nitrocellulose Blotting Membrane, GE Healthcare

Plasmids
- pEGFP-C1
- pEGFP-C1-RIG-I-CARD
- pEGFP-C1-RIG-I
- pEGFP-C1-RIG-I-Helicase
- pCMV-2A-RIG-I-CARD

Solutions and buffers
- Blotting Buffer: 0,05 M Tris, 0,4 M Glycine, 0,05% SDS, 20% Methanol
- Lysis Buffer: 25 mM Tris-HCl pH 8, 50 mM NaCl, 0,5% Sodium Deoxylacholat, 1,5 mM Triton X-100
    - Add protease inhibitors before use: 0,01 mM Foy, 0,25 (v/v) Trasylol, 0,1 mM PMSF
- Loading buffer: 1 M Tris pH 6.80, 10% Glycerol, 1 M DTT, 20% SDS, β-Mercaptoethanol, 0,1% Bromophenol Blue
- Milk for Blot blocking: 5 g of powder milk in 100 mL of 1x TBS-T
- 1x PBS: 1,37 M NaCl, 80 mM $NaHPO_4$, 0,0268 M KCl, 0,0147 M $KH2PO_4$ pH 7,34, prepared from 10x PBS diluted in ddH_2O
- Running Buffer 1x: 250 mM Tris, 1,9 M Glycine, 1% SDS
- TBS-T: 1X TBS-T (100 mM Tris Base, 150 mM sodium chloride and 0,05% Tween-20 [pH 8]).

Transfection Reagents
- Lipofectamine® 3000 Transfection Reagent, Invitrogen
- Lipofectamine RNAiMAX® Transfection Reagent, Invitrogen
- Screenfect®A, InCella

3.2 - Methods

Cell Culture

Mefs MAVS-PEX cells (with MAVS localized solely at the peroxisomes) were cultured in 10 cm culture dishes with DMEM (Life Technologies, Germany) supplemented with 10% FBS, 100 U/mL penicillin and streptomycin, and maintained in a humidified incubator with 5% CO_2 at 37°C.

Subculture was done in 10 cm culture dishes twice a week when the cells achieved the required confluence. At this point cells were washed with PBS and then incubated with 2 mL trypsin-EDTA for 30 seconds at 37°C and 5% CO_2 in order to be harvested. In this step 4 mL of cell culture medium was added and cells were centrifuged at 1000 rpm for 3 minutes at room temperature. Following cell pellet resuspension in 10 mL of DMEM cells were seeded in a 1:10 dilution (≈10^5 cells/mL).

Western Blotting

Mefs MAVS-PEX cells were cultured in 6-well plates. After 24 hours and transfected with Lipofectamine 3000 or Screenfect A. After 6 hours or 24 hours of transfection cells were collected in 200 µL lysis buffer and resuspended 20 times with a 1 mL syringe and a 26G needle. After this step, over-head rotation followed during 30 minutes at 4°C and then centrifugation to clear the lysate for 15 minutes at 13 000 rpm at 4°C.

Protein concentration was accessed using Bradford reagent (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, Hercules, California, USA). BSA standards were made in duplicate with known protein concentration (1 µg/µL BSA diluted in 0,1 M NaOH: 1- 11 µg) and 3 µL of each sample was diluted in 0,1 M NaOH. The Bradford reagent was diluted in dH_2O (1:5) and 1 mL was added to each tube followed by 15 minutes of incubation in the dark at room temperature. Samples absorbance was measured using a spectrophotometer at 595 nm and a standard curve was designed in Excel in order to achieve samples protein concentration values.

Loading buffer was added to 50 µg of samples and they were heated to 95ºC for 5 minutes. Proteins were concentrated in a 4% stacking polyacrylamide gel and separated in a 10% polyacrylamide gel (SDS-PAGE). The western blot system was filled with running buffer and the pre-stained protein marker was loaded in the gel as well as the samples. The electrophoresis was performed at 160 V for 1 hour and 30 minutes. Then a nitrocellulose membrane and filter paper were used for semi-dry or wet-blot. Foam pad, filter paper, gel, membrane, filter paper and foam pad were placed in this order in the cassette after incubating in transfer buffer. This buffer was used to fill the chamber and the blotting

driven at 0,4 A for 2 hours. When using the semi-dry instead, the transfer buffer was poured on the components without the cassette and the blotting was performed at 12 V, 0,4 A for 1 hour.

Membranes were blocked with 5% (w/w) low fat powder milk diluted in 1x TBS-T for 1 hour at room temperature and stained with primary antibodies at room temperature for 1 hour to 3 hours, and secondary antibodies were incubated at room temperature for 1 hour. Between all the steps membranes were washed with 1x TBS-T during 5 minutes for 3 times. The primary antibodies used were rabbit anti-RIG-I (1:200, Santa Cruz Biotechnology), rabbit anti-STING (1:500, Cell Signaling), mouse anti-α-Tubulin (1:4000, Sigma-Aldrich), and as secondary antibodies anti-rabbit IR Dye 800 CW (1:10000, Li-Cor), anti-mouse IR Dye 680 RD (1:10000, Li-Cor). Membranes were analysed with Odyssey and protein quantification was performed using Quantity One Software and Tubulin protein intensity was used as normalizer.

Small interfering RNA (siRNA)-mediated knockdown

The knock-down of STING was performed in Mefs MAVS-PEX cells using Lipofectamine RNAiMax reagent to transfect siRNA (5'-UCU CGU AGA CGC UGU UGG-3' and 5'-CCA ACA GCG UCU ACG AGA-3') in order to optimize the protocol different concentrations of siRNA were tested (25nM, 40nM, 45nM). After incubating for 20 min, the diluted siRNA was added to the suspended cells in DMEM supplemented with 10% FBS. Cells incubated for 48 hours before being harvested or stimulated with RIG-I constructs.

Transfection

- **Lipofectamine 3000**

For transfection with Lipofectamine 3000, a ratio of 1:1 (DNA: P3000) was used and 2 mixtures were needed: one with 90 µL OptiMem and 2,7 µL Lipofectamine 3000 and the other one with 90 µL OptiMem, 2,7 µL P3000 and 2,7 µg DNA (except in cGAMP experiments where cGAMP was transfected at a final concentration of 4 µg/mL). The second mixture was added to the first and incubated for 5 min at room temperature. The complex formed in this reaction was added dropwise to 6-well plates making a final volume of 2 mL and it was incubated for 6 hours or 24 hours. This method was used to transfect Mefs MAVS-PEX cells with GFP-RIG-I-CARD, GFP-RIG-I, GFP-RIG-I-Helicase, GFP-C1 and cGAMP.

- Screenfect A

For transfection with Screenfect, a ratio of 1:6 (DNA: Screenfect) was used and 2 mixtures were needed: one with 120 µL Dilution Buffer and 6 µL Screenfect and the other one with 120 µL Dilution Buffer and 1 µg DNA. The second mixture was added to the first and it was vortexed and incubated for 20 min at room temperature. The complex formed in this reaction was added dropwise to 6-well plates making a final volume of 1,5 mL and it was incubated for 6 hours. Screenfect A was used to transfect Mefs MAVS-PEX cells with GFP-RIG-I-CARD and cGAMP. For the cGAMP experiments, cGAMP was transfected at a final concentration of 4 µg/mL and ratios of 1:3 and 1:4 were tested.

IV. Results

IV. Results

The antiviral innate immune response may be activated through several distinct pathways depending on the type of viral nucleic acid. However, many authors suggest that these pathways may be interlinked and influence each other at some point.

The ER adaptor STING has been described as a key molecule in the antiviral signalling to dsDNA, acting on its own signalling pathway, independently from RIG-I-MAVS. As previously explained (Introduction chapter), cGAS, a recently identified cytosolic DNA sensor, binds dsDNA and catalyses the synthesis of cGAMP which, in turn, binds to STING, leading to the activation of a signalling cascade that culminates with the expression of IFN (Sun et al., 2013). Besides its independent role on antiviral signalling, STING is able to interact with RIG-I and mitochondrial MAVS, playing a role on the mitochondrial-dependent signalling pathway (Ishikawa and Barber, 2008; Zhong et al., 2008).

Although many studies have been performed related to the influence of the STING pathway on the mitochondria-dependent RIG-I/MAVS pathway, no study has ever reported its influence of the peroxisome-dependent pathway. Only recently peroxisomes have been highlighted as playing an important role on antiviral signalling and some recent reports indicate that the mechanisms involved may be somewhat different to the ones occurring in mitochondria (Dixit et al., 2010; Magalhães et al., 2016; Odendall et al., 2014).

Our group has previously performed co-immunoprecipitation analysis on Mefs cells that expressed MAVS solely at peroxisomes (Mefs MAVS-PEX cells) and found out that STING interacts with peroxisomal MAVS (unpublished data). Furthermore, this interaction is still observed in the presence of HCMV's vMIA (unpublished data). It has previously been shown that this viral protein is able to interfere with the interaction between STING and mitochondrial MAVS (Castanier et al., 2010). These results demonstrate once more the existence of important differences between the antiviral pathways established at these two organelles.

In this study we aimed at evaluating whether and how the STING pathway influences the peroxisomal RIG-I/MAVS pathway.

4.1 – Analysis of the importance of STING for the establishment of an effective peroxisomal MAVS-dependent antiviral response

In order to analyse the importance of STING for the establishment of an effective peroxisome-dependent antiviral response, we aimed at analysing the production of ISGs upon stimulation of the RIG-I/MAVS pathway in the absence or presence of STING.

To specifically analyse the peroxisomal RIG-I/MAVS pathway, these experiments were performed in Mefs cells that express MAVS solely at peroxisomes (Mefs MAVS-PEX cells) (Dixit et al., 2010).

4.1.1 Knock-down of STING by small interference RNA

In order to perform these studies in the absence of STING, we silenced STING via siRNA in Mefs MAVS-PEX cells, prior to stimulation of the RIG-I/MAVS pathway.

We initially performed several experiments to optimize the silencing conditions, using different concentrations of siRNA (from 25 to 45 nM) and different time points. After many trials and optimization steps, the best results that were obtained reflected a decrease in the expression of STING of about 79% (**Figure 4 and Table 1**).

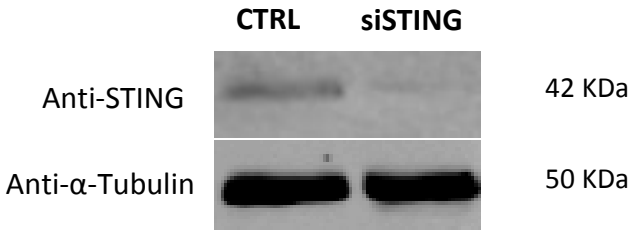

Figure 4 –Silencing of STING. Western blot analysis of the silencing of STING in Mefs MAVS-PEX cells.

Table 1 – Quantification of the silencing of STING observed in Figure 4.

	STING	Tubulin	STING/Tubulin	STING/CTRL	%	% STING decrease
CTRL	108,84	699,76	0,16	1	100	
siSTING	19,36	581,84	0,03	0,21	21	79

4.1.2 – Stimulation of the peroxisomal RIG-I/MAVS pathway in the presence and absence of STING

In order to stimulate the RIG-I/MAVS signaling pathway in Mefs MAVS-PEX cells, we used a methodology routinely performed in our laboratory that involves the transfection

of a constitutively active version of RIG-I (GFP-RIG-I-CARD) (Ferreira et al., 2016; Magalhães et al., 2016). This protein lacks the repressor helicase domain, having their CARD domains immediately accessible to interact with MAVS, even in the absence of viral infection.

Forty eight hours after silencing STING, the cells were transfected with GFP-RIG-I-CARD. Six hours post-transfection the cells were collected and analysed by Western blot with antibodies against RIG-I, STING and tubulin (**Figure 5**). Cells were also collected for mRNA extraction and RT-qPCR analysis of the production of ISGs mRNAs. In this way, we aimed at analysing whether the absence of STING would influence ISG production upon RIG-I/MAVS stimulation.

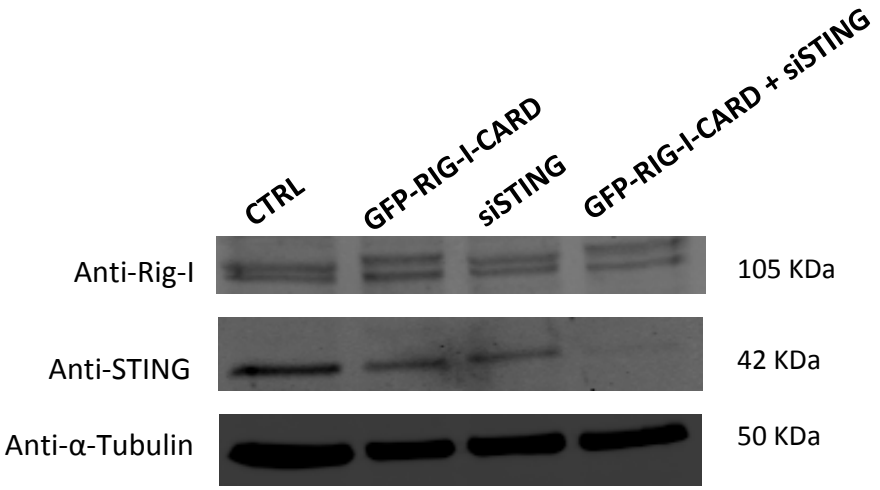

Figure 5 - STING silencing and GFP-RIG-I-CARD transfection. Western blot analysis of Mefs MAVS-PEX cells stimulated with GFP-RIG-I-CARD and upon silencing of STING. Non-silenced cells, as well as not stimulated cells were used as controls.

Table 2 – Quantification of the expression of STING observed in Figure 5.

	STING	Tubulin	STING/Tubulin	STING/CTRL	%	% STING decrease
CTRL	138,27	261,24	0,53	1	100	
siSTING	39,18	172,77	0,23	0,43	42,84	57
GFP-RIG-I-CARD	56,38	151,71	0,37	0,70	70,22	
GFP-RIG-I-CARD + siSTING	4,11	187,08	0,02	0,04	4,15	95,9

The Western blot analyses revealed unexpected results: there was a clear decrease in the amount of STING present in the cells that had been stimulated with GFP-RIG-I-CARD, both in non-silenced and in the silenced ones, when compared with the respective controls (**Figure 5** and **Table 2**). This result was obtained in the many times this experiment was repeated.

A first interpretation would suggest that stimulation with GFP-RIG-I-CARD would induce a decrease in STING production. However, it became necessary to perform further experiments with additional controls:

a) In order to check whether this decrease was actually due to a stimulation of the RIG-I/MAVS pathway, we transfected GFP-RIG-I, a full length RIG-I that is unable to interact with MAVS in the absence of viral RNA, hence, not inducing any activation of the antiviral pathway;

b) With the same purpose, we transfected GFP-RIG-I-Helicase, a mutant of RIG-I that contains solely the helicase domain, not being able to interact with MAVS and stimulate the signalling cascade;

c) In order to check that the GFP tag itself was not causing the observed effect, we transfected a Flag-tagged version of RIG-I-CARD, Flag-RIG-I-CARD;

d) In order to check whether the presence of transfected DNA (independently of RIG-I) would, by itself, induce the observed decrease in STING production, we transfected the GFP-C1 vector;

e) To exclude that the observed effect would be due to the transfection reagent, we performed the transfection of GFP-RIG-I-CARD with another transfection reagent (Screenfect);

f) Lastly, we compared the observed effect upon GFP-RIG-I-CARD transfection after 6 hours and 24 hours post-transfection.

There experiments were performed in Mefs MAVS-PEX cells and the Western blot results are shown in **Figure 6**.

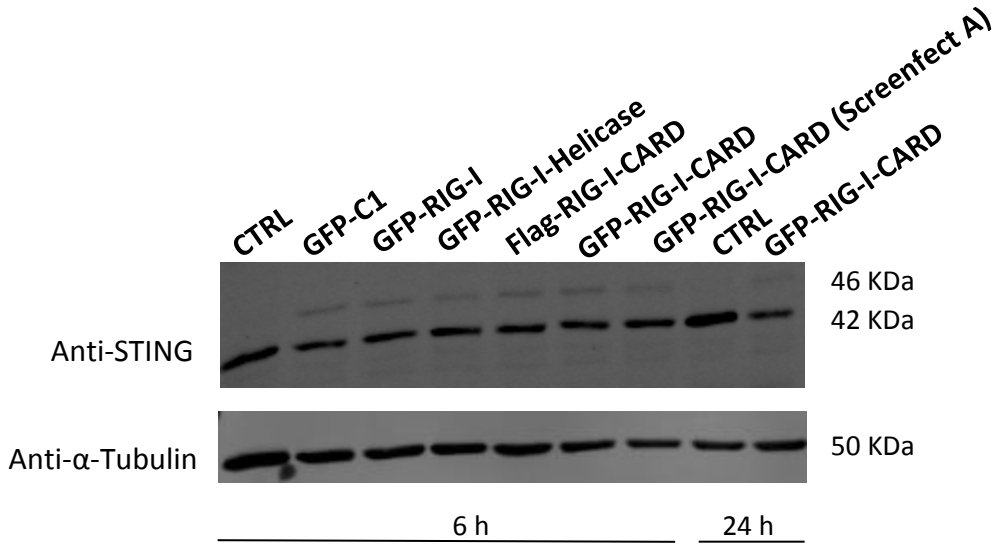

Figure 6 - Analysis of STING upon transfection with different DNAs. Western blot analysis of Mefs MAVS-PEX cells transfected with GFP-C1, GFP-RIG-I, GFP-RIG-I-helicase, Flag-RIG-I-CARD and GFP-RIG-I-CARD. Non-transfected cells were used as controls.

Table 3 - Quantification of the STING expression upon transfection with different DNAs observed in Figure 6.

		STING	Tubulin	STING/Tubulin	STING/CTRL	%	% STING decrease
6 h	**CTRL**	83,52	104,98	0,80	1	100	
	GFP-C1	58,48	125,50	0,47	0,59	59	41
	GFP-RIG-I	66,44	133,05	0,50	0,63	63	37
	GFP-RIG-I-Helicase	65,28	118,76	0,55	0,69	69	31
	Flag-RIG-I-CARD	65,48	121,91	0,54	0,68	68	32
	GFP-RIG-I-CARD	63,78	99,72	0,64	0,80	80	20
	GFP-RIG-I-CARD (Screenfect A)	64,96	87,37	0,74	0,93	93	7
24 h	**CTRL**	101,87	86,49	1,18	1	100	
	GFP-RIG-I-CARD	47,10	100,49	0,47	0,40	40	60

As shown in **Figure 6** and **Table 3**, the transfections with all the DNAs induced a decrease on the amount of STING present in the cells. This experiment was repeated and these results were confirmed. We have also observed the appearance of a new 46 KDa band that is recognized by the STING antibody. This band seems to correspond to the

phosphorylated version of STING (Hu et al., 2016; Ma et al., 2015a; Wang et al., 2016). These results indicated that the simple presence of transfected DNA in the cells is activating the STING pathway in a significant manner. Although we naturally expected that the presence of foreign DNA would somehow induce the activation of this pathway, we were surprised to observe that, in these cells, this occurs in such high levels. In this way, it was not possible to proceed with this methodology and we decided to use a different stimulus of the RIG-I/MAVS pathway that would not involve insertion of foreign DNA. We initiated optimization experiments for the use of poly(I:C) (a viral RNA mimic) but unfortunately, due to time constraints, it was not possible to obtain the results on time to be presented in this thesis.

4.2 - Analysis of the peroxisomal MAVS-dependent antiviral response upon stimulation of the STING pathway

Besides the study of the importance of STING for the establishment of an effective peroxisomal MAVS-dependent antiviral response, we aimed at studying the effect on this response of the direct and specific stimulation of the STING pathway.

To that end, we used 2'3'-cGAMP, which binds directly to STING, leading to its dimerization and triggering IFN production (Kemp et al., 2015; Ma et al., 2015b).

In order to specifically optimize the delivery of 2'3'-cGAMP to Mefs MAVS-PEX cells, we performed different transfections with several 2'3'-cGAMP concentrations and using two different transfection reagents (Lipofectamine 3000 and Screenfect A). We have also analysed the delivery of 2'3'-cGAMP in the absence of transfection reagents, adding the DNA directly in the medium. Cells were collected after 6 hours and Western blot analyses were performed with antibodies against STING and tubulin (a representative example is shown in **Figure 7**).

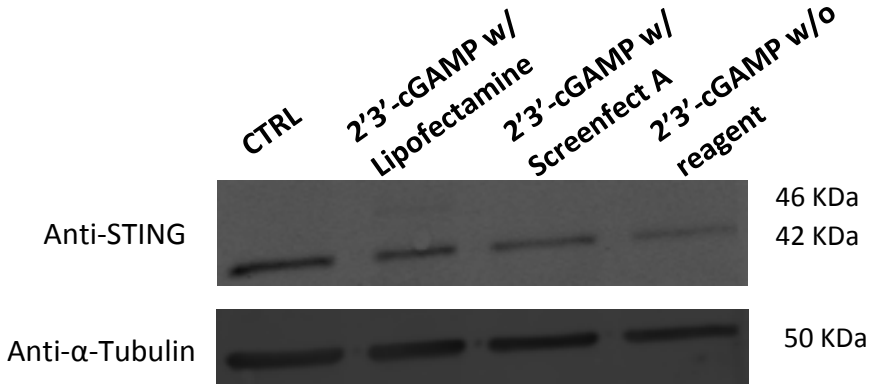

Figure 7 - Transfection of 2'3'-cGAMP in Mefs MAVS-PEX cells. Western blot analysis of MAVS-PEX cells transfected with 2'3'-cGAMP using Lipofectamine 3000, Screenfect A and by simple addition to the medium in the absence of a transfection reagent.

The data shown in **Figure 7** demonstrates that none of the methodologies used resulted in a strong stimulation of the STING pathway. Only with the use of Lipofectamin 3000 a faint band likely corresponding to the phosphorylated STING can be observed.

Further optimizations are currently being performed but, due to time constraints, it was not possible to include the results in this thesis.

Once a proper stimulation of the STING pathways is obtained, the mRNA of the cells will be quantified by RT-qPCR and the production of ISGs via the peroxisome-dependent RIG-I/MAVS pathway will be analysed.

V. Discussion

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The innate immune system is the organism's first line of defence against pathogens. Viral RNA and DNA are recognized by several different cellular sensors, such as RIG-I and cGAS, which activate signalling cascades that culminate with the production of IFN and other cytokines that interfere with the virus life cycle and hamper spreading to other cells.

Although most known antiviral signalling pathways have been studied in detail in recent years, there is still some controversy on how these pathways interact with and influence each other. Even though the RIG-I/MAVS and the STING pathways are assumed to signal, respectively, for RNA and DNA viruses, some authors defend that the complete signalling against viral RNA also relies on STING (Ishikawa and Barber, 2008; Nazmi et al., 2012; Zhong et al., 2008). Moreover, different RNA viruses have developed tools to evade the STING signalling (Aguirre et al., 2012; Ding et al., 2013; Sun et al., 2012). Furthermore, an activation of the RIG-I pathway by DNA viruses is also described by several authors, who defend that cytosolic AT-rich dsDNA is transformed into 5'-ppp RNA by RNA polymerase III resulting in the activation of RIG-I signalling (Ablasser et al., 2009; Chiu et al., 2009; Härtlova et al., 2015).

The interaction between STING and MAVS (Ishikawa and Barber, 2008; Zhong et al., 2008) also supports a crosslink between the STING and RIG-I/MAVS pathways. This interaction was reported to occur at mitochondria, where MAVS was initially localized. In recent years, this protein was also found to localize at the peroxisomes (Dixit et al., 2010) and the MAM (Horner et al., 2011). Peroxisomal and mitochondrial MAVS perform different but complementing functions within the antiviral response: while the peroxisomal MAVS induces the rapid expression of defence factors, providing short-term protection, the mitochondrial MAVS activates a signalling pathway with delayed kinetics that amplifies and stabilizes the antiviral response (Dixit et al., 2010; Odendall et al., 2014).

Our group has recently demonstrated that STING is also able to interact with the peroxisomal MAVS (unpublished data). This interaction was shown to occur even in the presence of vMIA, a protein from HCMV that had been previously shown to disrupt the interaction between STING and the mitochondrial MAVS (Castanier et al., 2010). Hence, although these two organelles share many functions and proteins, there seems to be important differences concerning the establishment of the antiviral signalling pathways.

With this work we aimed at studying in more detail the interplay between the STING pathway and the peroxisomal RIG-I/MAVS pathway. Our first approach involved the knock-down of STING and stimulation of the RIG-I/MAVS pathway in cells that contained MAVS solely at peroxisomes. In this way, we could study the importance of STING for the establishment of an effective peroxisome-dependent antiviral response. We decided to apply a methodology routinely used in our laboratory that involves the transfection of GFP-RIG-I-CARD, leading to the expression of a constitutively active mutant of RIG-I which will

interact with MAVS and activate the antiviral signalling (Ferreira et al., 2016; Magalhães et al., 2016). Surprisingly, we observed a decrease in STING expression in all samples whose cells had been transfected with GFP-RIG-I-CARD. In order to understand whether this was due to the activation of the RIG-I/MAVS pathway, we performed transfections of these cells with plasmids expressing the inactive forms of RIG-I, GFP-RIG-I and GFP-RIG-I-Helicase. As the results obtained were similar to the ones obtained with the GFP-RIG-I-CARD or even the Flag-RIG-I-CARD, we analysed the levels of STING upon expression of the empty GFP-C1 vector. These results confirmed that, in these specific cells, a simple transfection of DNA activates the STING pathway. This activation was confirmed by the presence, in all these experiments, of a band of about 46 KDa that represents phosphorylated STING (Hu et al., 2016; Ma et al., 2015a; Wang et al., 2016).

As we could not, hence, use this methodology to activate the peroxisomal RIG-I/MAVS pathway, we initiated the optimization of the transfection of poly(I:C), a viral RNA mimic. However, due to time constraints, these results are not included in this thesis.

In parallel, we followed another approach to study the influence of the STING pathway on the peroxisomal RIG-I/MAVS pathway: activation of STING by transfecting 2'3'-cGAMP and analysis by RT-qPCR of the peroxisome-dependent production of ISGs. We initiated these procedures with the optimization of the transfection of Mefs MAVS-PEX cells with 2'3'-cGAMP by using different concentrations and transfection reagents. Although none of the conditions resulted in a strong stimulation of the STING pathway, with the use of Lipofectamin 3000 a faint band likely corresponding to the phosphorylated STING can be observed. Further optimizations are currently being performed.

Both approaches presented in this study are currently being followed up in our laboratory and results should soon arise, demonstrating whether or not these two pathways interact with each other. If the obtained results would reflect what has been reported for mitochondrial MAVS, one would expect to observe an increase on the host defence responses to RNA viruses upon stimulation of STING with 2'3'-cGAMP in Mefs MAVS-PEX cells. Similarly, one would expect a decrease on the production of peroxisome-dependent antiviral factors upon stimulation with poly(I:C) in the absence of STING. However, as noteworthy differences between the peroxisome and mitochondria-dependent antiviral pathways have been discovered, also specifically concerning the STING-MAVS interaction, it is not yet possible to further discuss about the expected results.

The interaction between cGAMP and STING induces the phosphorylation of TBK1 leading to the activation of IRF3 and its consequent translocation to nucleus (Abe and Barber, 2014), followed by IFN expression. TBK1 was found to localize in mitochondria upon Hepatitis B virus or Herpes simplex virus infection (Suzuki et al., 2013). It would be interesting to analyse whether TBK1 is also localized at peroxisomes, upon viral infection or stimulation with poly(I:C) or 2'3'-cGAMP.

The Dengue virus encodes the protease complex NS2B3 which cleaves STING and inhibits IFN production (Yu et al., 2012). It was also shown that NS4B from Hepatitis C virus

disrupts the interaction between STING and TBK1 suppressing IFN production (Ding et al., 2013). The overexpression of these proteins in Mefs MAVS-PEX cells (and consequent disruption of the STING pathway in two different stages) and analysis of the production of ISGs would allow us to analyse which, if any, of these stages is relevant for the peroxisome-dependent signalling pathway.

The studies initiated with this master thesis will certainly contribute to unravel the interplay between the STING pathways and the peroxisomal-dependent RIG-I/MAVS signalling.

VI. References

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